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Characterization and distribution of invertase activity in developing maize *(Zea mays)* kernels

Douglas C. Doehlert and Frederick C. Felker

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Invertase (β-fructofuranoside fructohydrolase, EC 3.2.1.26) activity in developing maize (*Zea mays* L. inbred W64A) was separated into soluble and particulate forms. The particulate form was solubilized by treatment with 1 *M* NaCl or with other salts. However, $CaCl_2$ inhibited invertase activity, and neither detergents nor 0.5 *M* methyl mannoside were effective in solubilizing the invertase activity. The soluble and particulate invertases were both glycoproteins, both had pH optima of 5.0 and K_m values for sucrose of 2.83 and 1.84 m*M*, respectively. The apparent molecular weight of salt-solubilized invertase was 40 kDa. Gel filtration of the soluble invertase showed multiple peaks with apparent molecular weights ranging from 750 kDa to over 9 000 kDa. Histochemical staining of cell wall preparations for invertase activity suggested that the particulate invertase is associated with the cell wall. Also, nearly all the invertase activity was localized in the basal endosperm and pedicel tissues, which are sites of sugar transport. No invertase activity was found in the upper endosperm, the embryo or in the placento-chalazal tissue. In contrast, sucrose synthase (EC 2.4.1.13) activity was found primarily in the embryo and the upper endosperm, which are areas of active biosynthesis of storage compounds.

Additional key words – Corn, β-fructosidase, invertase histochemistry, sucrose.

D. C. Doehlert (reprint requests) and F. C. Felker, Northern Regional Research Center, Agricultural Research Service, U.S. Dept of Agriculture, 1815 N. University St., Peoria, IL 61604, U.S.A.

Introduction

The developing maize (*Zea mays* L.) kernel is dependent on a supply of phloem-transported sucrose for growth. The incoming sucrose may be initially hydrolyzed by invertase (β-fructofuranoside fructohydrolase EC 3.2.1.26) into glucose and fructose, or sucrose may be metabolized by sucrose synthase (EC 2.4.1.13), which is also present in maize kernels (Tsai et al. 1970).

Invertase was reported to be present in the pedicel and placento-chalazal tissues of developing corn kernel by Shannon and Dougherty (1972). They reported finding soluble and particulate forms of invertase, both with pH optima at pH 5.0. The presence of high concentrations of reducing sugars in the basal region of the kernel suggested that the invertase activity in this region readily hydrolyzed sucrose arriving from the phloem.

Jaynes and Nelson (1971) partially characterized invertase activity in maize endosperm. They described three forms of invertase, two soluble (invertase I and II) and one particulate (invertase III). Their invertase I was present in endosperms from 10 to 28 days after pollination, whereas invertase II was present in germinated embryos and in endosperms harvested before 6 days after pollination. Invertases I and II had K_m values for sucrose of 2.0 and 9.7 m*M*, respectively. The two soluble invertases were separable by both ion-exchange chromatography and gel filtration, with invertase I being very high in molecular weight. They reported a K_m value for sucrose of 8.7 m*M* for the particulate invertase III.

Many investigators studying particulate invertases have provided evidence that it is associated with the cell wall. Burström (1957, 1958) demonstrated that excised roots hydrolyze external sucrose in a liquid medium. Kivilaan et al. (1959, 1961) showed an association of inver-

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tase activity with their cell wall preparations. Ueda et al. (1974) found that the formation of carrot protoplasts by the action of cellulase and pectinase was accompanied by the release of 50 to 60% of the invertase activity from the cells. Perhaps the strongest evidence suggesting the association of invertase with cell walls was provided by Faye and Ghorbel (1983a). They prepared antibodies to insoluble invertase from radish seedlings and determined through immunocytochemical techniques that this enzyme was associated with the cell walls of the radish hypocotyl. The cell wall-bound invertase would presumably hydrolyze apoplastic sucrose, which may be a prerequisite for carbohydrate uptake into the symplast by maize endosperm cells (Shannon 1968).

Although Jaynes and Nelson (1971) reported that particulate invertase from maize endosperm could not be solubilized by salt or solvents, other investigators have found that insoluble invertase from Jerusalem artichoke tuber, sugar beet seedlings, radish seedlings and strawberry fruit could be solubilized by treatment with salt (Little and Edelman 1973, Masuda and Sugawara 1978, 1980, Faye and Ghorbel, 1983b, Poovaiah and Veluthambi 1985).

In the present report results on the distribution and characteristics of invertase activity in developing maize kernels are reported in order to evaluate the possible role of this enzyme in sucrose metabolism.

Materials and methods

Plant material

Maize (*Zea mays* L. inbred W64A) was grown in the field in the summer of 1984, pollinated by hand and harvested 22 days after pollination. The ears were frozen and stored at −70°C until use. Kernels used in histochemical studies were grown in a greenhouse in the winter of 1985–86. Kernels were dissected with a razor blade. The pericarp separated easily. The basal endosperm separated from the lower pericarp at the placento-chalazal tissue. The embryo was removed with forceps and the endosperm was cut into lower (basal), middle and upper (crown) thirds.

Enzyme extraction

Tissue was homogenized in 10 ml extraction buffer (g fresh weight)$^{-1}$ with a Brinkman Polytron. Extraction buffer contained 50 m*M* Tris-maleate (pH 7.0) and 1 m*M* dithiothreitol. Crude homogenates were centrifuged at 27 000 *g* for 15 min to separate the soluble invertase (supernatant) from the particulate invertase (pellet). The pellets were washed 3 times by resuspending in extraction buffer and centrifuging at 27 000 *g* for 15 min. Particulate invertase was extracted by resuspending the pellet in extraction buffer containing 1 *M* NaCl. Solubilized particulate invertase was recovered in the supernatant following centrifugation at 27 000 *g* for 15 min.

Enzyme assays

Invertase (EC 3.2.1.26) activity was assayed for 10 to 30 min at 30°C in a mixture containing 200 m*M* Na-acetate (pH 5.0) and 10 m*M* sucrose in a final volume of 100 µl. Activity was determined by measuring either glucose produced by means of the glucose oxidase method (Gascon and Lampen 1968) or measuring reducing sugars with the method of Nelson (1944). One unit of activity is defined as the activity required to hydrolyze 1 µmol sucrose in 1 min at 30°C, pH 5.0.

Sucrose synthase (EC 2.4.1.13) activity was assayed by determining sucrose produced in the presence of 12 m*M* fructose and 12 m*M* uridine-5′-diphosphoglucose in 50 m*M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES, pH 7.5) at 30°C by the procedure of Cardini et al. (1955). Protein was determined with Bio-Rad protein reagent with ovalbumin as a standard.

Chromatography

Salt-solubilized particulate invertase was fractionated on a 1×60 cm Ultrogel AcA 54 (LKB, Bromma, Sweden) column equilibrated with 50 m*M* Tris-maleate (pH 7.0) containing 1 m*M* dithiothreitol and 500 m*M* NaCl. Soluble invertase was fractionated on a 1×60 cm Ultrogel A-7 column equilibrated with the same buffer containing no NaCl. Both soluble and particulate invertase were fractionated on a 2×8 cm concanavalin-A agarose column equilibrated with 50 m*M* tris-maleate (pH 7.0) containing 1 m*M* dithiothreitol and 500 m*M* NaCl. Invertase activity was eluted with the equilibration buffer containing 200 m*M* methyl mannoside.

Cell wall preparations

Cell walls were prepared by grinding 2 to 3 g of lower endosperm tissue using a mortar and pestle. This material was filtered through a 150 µm Nitex cloth. The material remaining on the cloth was washed several times in water. This large particulate fraction was resuspended in water and homogenized for 2 s in a Brinkman Polytron. This was filtered again through a 150 µm Nitex cloth and washed extensively. The large particulates

Tab. 1. Extraction of particulate invertase from washed endosperm pellet by NaCl. Values are the mean of three extractions ± SD.

NaCl, *M*	Invertase activity, units (g FW)$^{-1}$	Protein, mg (g FW)$^{-1}$
0	0	1.4±0.4
0.25	0.37±0.09	3.5±0.8
0.50	0.57±0.11	5.4±1.0
1.00	0.81±0.17	7.5±1.5
2.00	0.70±0.22	6.3±1.6
3.00	0.51±0.11	6.2±1.0

remaining on the cloth were resuspended in water. This preparation was sonicated for 3 min and again filtered, washed and resuspended in water.

Histochemical staining

Histochemical staining for invertase activity was carried out by a modification of the procedure described by Dahlqvist and Brun (1962). Whole kernels were longitudinally sectioned by hand with a razor blade and sections were fixed in 4% formalin (pH 7.0) for 30 min. Sections were then rinsed in water at least 10 times over a period of 3 to 5 h to remove all endogenous sugars. Sections were incubated for 20 min at room temperature in a reaction mixture containing 0.38 *M* sodium phosphate (pH 6.0), 0.24 mg ml^{-1} nitro blue tetrazolium, 0.14 mg ml^{-1} phenazine methosulfate, 25 units ml^{-1} glucose oxidase and 5 mg ml^{-1} sucrose. Control sections were incubated with the reaction mixture without sucrose. After rinsing in water, sections were postfixed in 4% formalin (pH 7.0) for 15 min followed by several rinses in water. Sections were stored in 15% ethanol at 4°C and later photographed through a dissecting microscope.

For light microscope histochemistry, longitudinal sections ca 1 mm thick were fixed as stated above, rinsed in water, embedded in Ames O.C.T. compound (Ames Co. Elkhart, IN, U.S.A.) and frozen for cryostat sectioning. Frozen sections (20 μm) were collected on gelatin-coated microscope slides, air dried, rinsed in several changes of water and incubated in the reaction mixture described above for 30 to 60 min. Slides were incubated horizontally in glass Petri dishes with frequent agitation. After rinsing with water, sections were mounted in glycerin jelly and photographed with a Zeiss photomicroscope.

Cell wall preparations were incubated in the reaction mixture for 20 min. The reaction was terminated by washing the cell walls in 95% ethanol. After several changes of 100% ethanol and xylene, the preparations were mounted on slides in Permount for photomicrography.

Results

Extraction of particulate invertase

The particulate invertase could be solubilized by treatment with salt solutions. Treatment of washed maize endosperm pellets with increasing concentrations of NaCl up to 1 *M* NaCl resulted in increasing solubilization of invertase activity and protein (Tab. 1). Treatment with NaCl at concentrations greater than 1 *M* resulted in solubilization of less activity. The invertase assay was not affected by NaCl concentrations up to 3 *M*, and extracts dialyzed into extraction buffer still showed more activity extracted with 1 *M* NaCl than 3 *M* NaCl (data not shown). The amount of invertase solubilized did not vary with the amount of time the pellet was suspended in 1 *M* NaCl. Various salts and other substances were tested for their effectiveness in solubilizing the particulate invertase. Most salts of either monovalent or divalent cations were effective with the exception of $CaCl_2$ (Tab. 2). Ca^{2+} was found to inhibit invertase at concentrations over 100 m*M* (data not shown), which accounts for the low invertase activity in those extractions. KCl and $(NH_4)_2SO_4$ were just as effective as NaCl in solubilizing the particulate invertase. Neither detergents (Triton X-100 or cholic acid) nor 0.5 *M* methyl mannoside solubilized invertase. These results suggest that the particulate invertase is probably bound to fragments by ionic interactions.

Properties of soluble and particulate invertase

Although the two forms of invertase were distinct with respect to their solubilities, in most other respects they had very similar properties. Both had optimal activity at pH 5.0, and they adsorbed onto concanavalin-A agarose columns and could be eluted with 200 m*M* methyl mannoside. Thus, both the soluble and particulate invertases appear to be glycoproteins. Soluble invertase, which had been partially purified by chromatography on concanavalin-A agarose, had a K_m for sucrose of 2.83 m*M*, whereas particulate invertase which had been partially purified by the same procedure after salt sol-

Tab. 2. Effectiveness of various solutions in the extraction of invertase from washed maize endosperm pellets. Buffer contained 50 m*M* Tris-maleate, pH 7.0, with 1 m*M* dithiothreitol. Values are the mean of three extractions ± SD.

Solution	Invertase activity, units (g FW)$^{-1}$
Buffer	0.014±0.005
1.0 *M* NaCl	0.68 ±0.12
1.0 *M* KCl	0.77 ±0.21
0.5 *M* $CaCl_2$	0.008±0.003
0.5 *M* $MgCl_2$	0.52 ±0.06
0.5 *M* $(NH_4)_2SO_4$	0.68 ±0.19
0.5 *M* methyl mannoside	0.035±0.012
0.1% Triton X-100	0.013±0.004
0.1% cholic acid	0.025±0.009

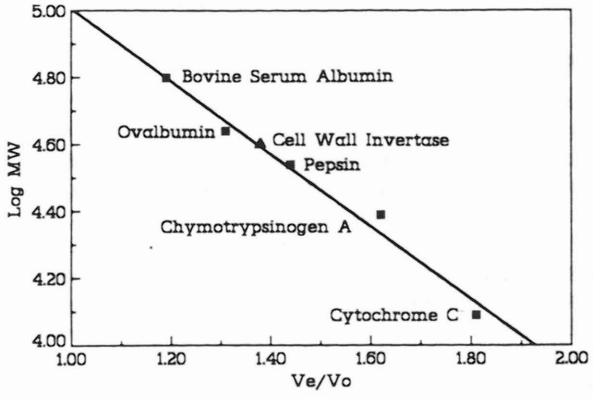

Fig. 1. Molecular weight estimation of salt-solubilized particulate invertase by gel filtration on Ultrogel AcA 54. The equilibration and elution buffer was 10 m*M* Tris-maleate (pH 7.0), containing 1 m*M* dithiothreitol and 500 m*M* NaCl. MW, molecular weight.

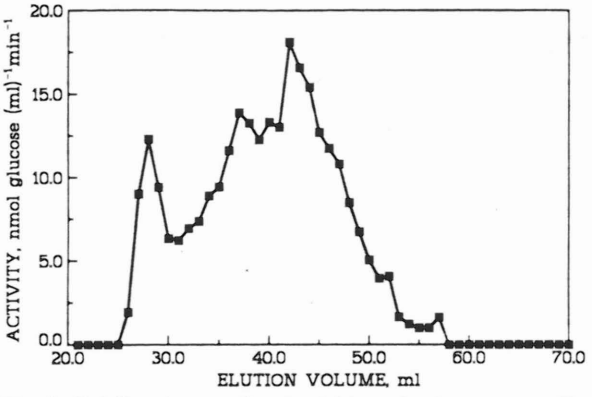

Fig. 2. Gel filtration profile of soluble maize invertase on Ultrogel A-7 column. The equilibration and elution buffer was 10 m*M* Tris-maleate (pH 7.0) containing 1 m*M* dithiothreitol. The void volume of the column was 28 ml.

ubilization had a K_m of 1.84 m*M*. Neither the K_m nor the pH optimum of the particulate invertase was affected by solubilization. Both enzyme preparations hydrolyzed either sucrose or raffinose but not melezitose or α-methyl glucose confirming that these are β-fructofuranosidases and not glucosido-invertases (Myrbäck 1960). The molecular weight of the particulate invertase was estimated to be 40 kDa by gel filtration on a calibrated Ultrogel AcA 54 column (Fig. 1).

Soluble invertase was not stable in salt solutions and, therefore, could not be chromatographed under the same conditions as could the solubilized particulate invertase. Soluble invertase eluted in the void volume of an Ultrogel AcA 34 column (data not shown) indicating an apparent molecular weight of over 750 kDa. The gel filtration profile on an Ultrogel A-4 column, with an exclusion limit of 9 000 kDa, still showed a portion of the soluble invertase eluting with the void volume, whereas the remaining activity was distributed throughout the fractionation range of this gel (Fig. 2). The addition of 0.1% Triton X-100 to a soluble invertase aliquot prior to gel filtration did not affect the elution profile, indicating that the soluble invertases are not associated with vesicles in the extract.

Distribution of invertase activity in the maize kernel

Soluble and particulate invertase activities of homogenates of various kernel tissues are shown in Tab. 3. The basal third of the endosperm contained the majority of the activity, although the lower pericarp also had considerable activity. The upper endosperm and the embryo contained no detectable activity. Soluble invertase comprised only 20 to 40% of the total activity in the kernel, whereas insoluble invertase comprised 60 to 80% of the activity. Sucrose synthase was present in the highest activities in the upper endosperm and in the embryo, tissues in which invertase activity was absent.

Histochemical staining of whole kernel sections for invertase activity showed a pattern similar to that seen in the extracts of dissected tissue (Fig. 3). Again the majority of the invertase activity was found in the basal endosperm and in the pedicel tissue. Of particular interest is the absence of invertase activity in the placento-chalazal tissue, which is a layer of dead cells between the pedicel and basal endosperm tissues. It has been previously suggested that this tissue contains high invertase activity, based on work with dissected tissue (Shannon and Dougherty 1972).

Association of particulate invertase with the cell walls

Cell wall preparations derived from basal endosperm tissue stained positively for invertase in the presence of sucrose (Fig. 4), but did not stain in the absence of su-

Tab. 3. Distribution of soluble invertase, particulate invertase and sucrose synthase activities in developing maize kernels. All values are a mean of three extractions with SD of 10 to 30% of the mean. nd, not detected.

Tissue	Units (g FW)$^{-1}$		Sucrose synthase, µmol sucrose (g FW)$^{-1}$ min^{-1}
	Soluble invertase	Particulate invertase	
Pericarp			
Lower	0.48	1.41	nd
Upper	nd	0.005	0.30
Embryo	nd	nd	3.60
Endosperm			
Lower	2.37	3.72	2.04
Middle	0.004	0.003	4.94
Upper	nd	nd	5.54

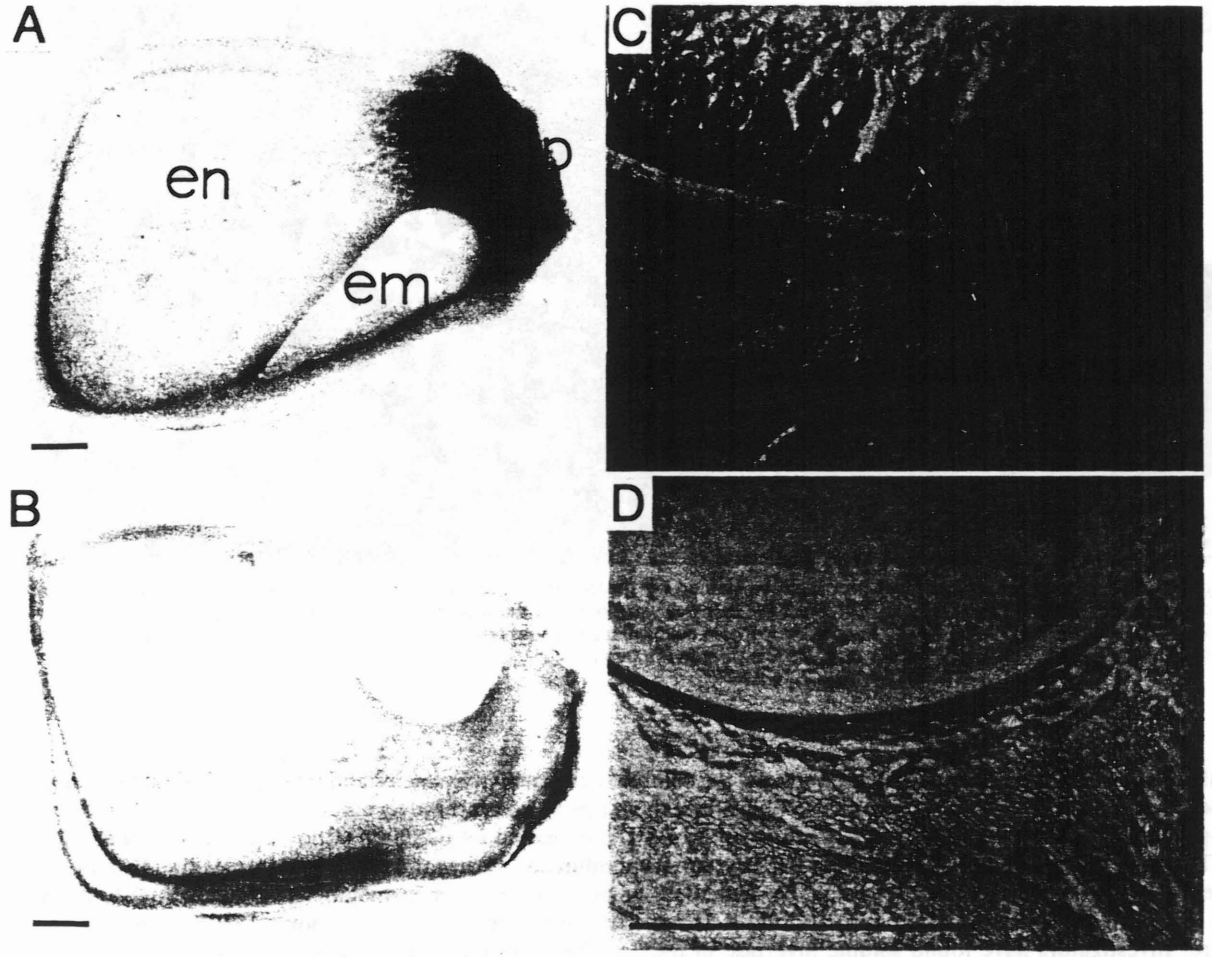

Fig. 3. Histochemical localization of invertase activity in longitudinal sections of maize kernels harvested 24 days after pollination. Bars represent 1 mm. A, 0.5 mm freehand section incubated in reaction mixture containing sucrose. Only basal endosperm and pedicel tissues contain blue formazan reaction product (black areas). Light grey areas correspond to pink unreacted dye. B, section immediately adjacent to that shown in A, control (− sucrose). No blue formazan reaction product is present. C, 20 μm frozen section (+ sucrose). D, as in C, control (− sucrose). No reaction product. em, embryo; en, endosperm; p, pedicel; pc, placentochalazal tissue; tc, transfer cells of basal endosperm.

crose. Cell walls that had been washed in 1 *M* NaCl prior to histological staining showed no reaction (data not shown). These results suggested that the particulate invertase was bound to the cell walls by ionic interactions.

Discussion

The restriction of invertase activity to the basal portions of the kernel suggests a role for invertase in the unloading of sucrose from the phloem and the subsequent uptake into the basal endosperm. Sucrose synthase, which is present in the endosperm and embryo (Tab. 3), may be more important in the conversion of sucrose to starch or other storage polymers. The apoplastic location of the cell wall invertase is consistent with its hypothesized role in sucrose unloading. Sucrose could be unloaded from the phloem into the apoplast and then hydrolyzed to glucose and fructose, thus maintaining a sucrose concentration gradient between the phloem and the apoplast. This concentration gradient would facilitate further passive sucrose unloading from the phloem and would thus favor sucrose movement into the endosperm, which presumably is actively taking up sugars via the basal endosperm transfer cells (Felker and Shannon 1980).

The invertases described here appear to be similar to the invertase isozymes I and III described by Jaynes and Nelson (1971). We found no invertase II in our 22-day-old kernels, which is consistent with Jaynes and Nelson (1971), who reported that invertase II was present in maize endosperm only prior to 6 days after pollination. Whereas we found a similar K_m for sucrose for the soluble invertase as did Jaynes and Nelson (1971) for invertase I, our particulate invertase had a lower K_m (1.8 m*M*) than their bound invertase (8.7 m*M*). The

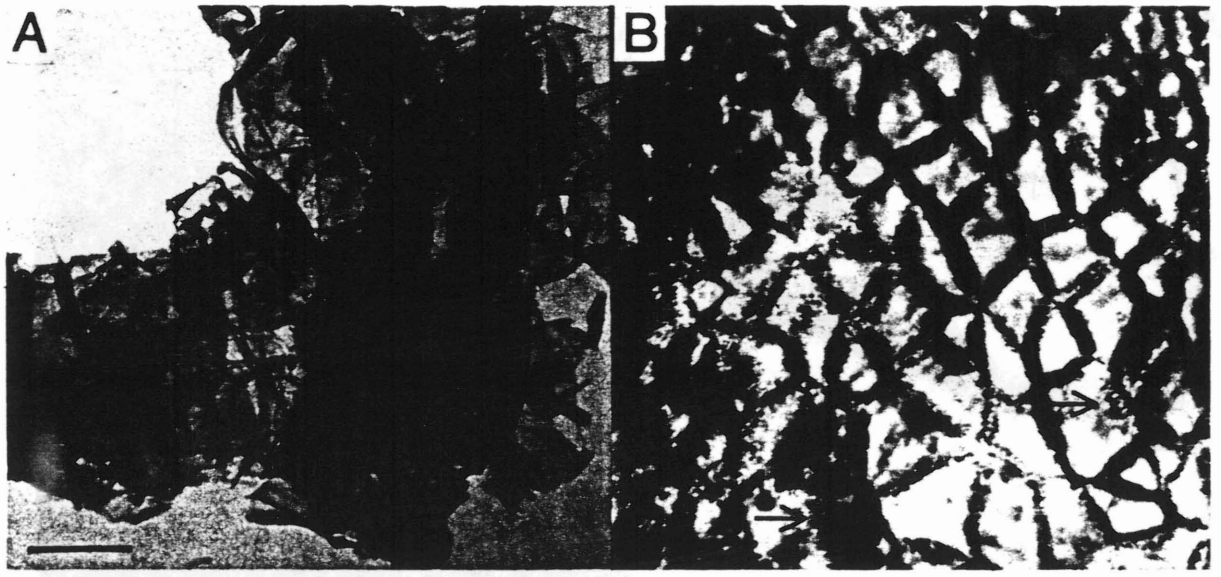

Fig. 4. Basal endosperm cell wall preparations stained by the histochemical reaction for invertase. Controls (− sucrose, not shown) and cell walls washed with 1 *M* NaCl (+ sucrose) were devoid of stain and therefore not visible using bright field microscopy. Bars represent 0.1 mm. A, smooth endosperm cell walls. Fragment contains walls of many cells. B, fragment consisting of basal endosperm transfer cells with striated wall ingrowths (arrows).

only obvious differences we found between the soluble and particulate invertases were their solubility and their apparent molecular weights.

The role of the soluble invertase is not clear from these experiments. Its function would be dependent on its intracellular localization, which was not determined here. Investigators have found soluble invertase in the vacuoles of tobacco cell suspension cultures, sugar beet storage root and sweet clover leaf mesophyll tissue (Boller and Kende 1979, Leigh et al. 1979, Boudet et al. 1981). Roberts (1973) found that soluble wheat leaf invertases were heterogeneous with respect to their isoelectric points and obtained gel filtration profiles similar to those presented here (Fig. 2), with molecular weights ranging from 200 kDa to over 7 000 kDa. It is possible that the apparent heterogeneity in the apparent molecular weights of the maize kernel soluble invertase were caused by differences in glycosylation and aggregation. Glycosylation has been shown to promote aggregation properties of yeast invertase (Chu et al. 1983), and such aggregation could result in the large forms of invertase found in this study (Fig. 2). Jaynes and Nelson (1971) also found that the soluble maize invertase I eluted in the void volume of a Sephadex G-200 column, suggesting that it had a molecular weight of over 200 kDa.

The presence of high hexose concentration in the basal portions of the maize kernel (Shannon 1972) clearly suggests that invertase activity is hydrolyzing sucrose in that tissue. The importance of sucrose synthase in sucrose metabolism is indicated by the shrunken 1 endosperm mutant of maize, which contains normal invertase activity (Jaynes and Nelson 1971) but lacks normal sucrose synthase levels and makes a fraction of the normal amount of starch (Chourey and Nelson 1976). The failure of invertase to compensate for the lack of sucrose synthase in metabolizing sucrose towards starch suggests it may not be so important in this function, but rather may function to facilitate phloem unloading as suggested here.

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